Aberrant Methylation of Heparan Sulfate Glucosamine 3-*O*-Sulfotransferase 2 Genes as a Biomarker in Colorectal Cancer

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Abstract. *Aim: The first aim was to determine whether hypermethylation of certain tumor related genes, including 3OST2, CHFR, RUNX3, and p16, could be detected in Japanese colorectal cancer (CRC) patients. The second was to utilize the most frequently hypermethylated genes as biomarkers for early detection of CRC. Materials and Methods: We investigated the aberrant promoter methylation profile of 4 genes in 129 colorectal tumors and corresponding normal mucosa. For the second step, bowel lavage and blood of the main tumor drainage vein from 21 CRC patients were analyzed. Results: The 3OST2 gene was the most frequently methylated (57%) and was methylated frequently (9/21, 43%) in bowel lavage but rarely (1/21; 4.7%) in the venous drainage. The concordance between 3OST2 methylation in bowel lavage and corresponding tumor samples was 71.4%. Conclusion: These results may show the possibility of early detection for CRC by examining 3OST2 gene methylation status in DNA extracted from stool.*

Colorectal cancer (CRC) is one of the most common types of cancer throughout the world. The annual age-adjusted incidence rates among men and women for CRC in Japan have increased rapidly over the past decade. CRC is the third leading cause of cancer in Japan, with over 22,000 men and 18,000 women dying from CRC yearly (1).

It is known that the majority of CRCs have truncating mutations or deletions of the *APC* gene or mutations of the

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β-catenin gene. Point mutations of *KRAS* proto-oncogene and mutation of the *P53* gene are also common. In a second pathway to colorectal neoplasia, microsatelite instability is caused by alteration of a nucleotide mismatch repair gene (2). Other molecular changes commonly present in CRCs are epigenetic abnormalities, including DNA methylation and histone modifications. Methylation occurs in the promoter region of many tumor suppressor genes in CRC cancers (3-7).

Promoter CpG island hypermethylation is an important epigenetic change associated with gene silencing and is recognized as an alternative mechanism to mutations for the inactivation of tumor suppressor genes or tumor-related genes in many types of cancer (8). It is now recognized that methylation can affect the critical pathways that regulate cell cycling, apoptosis, DNA repair, growth and invasion (8, 9). This phenomenon occurs early during CRC development (10, 11), supporting the possibility of methylation as a biomarker for early detection of CRC, as evidenced by multiple studies (12-14). Examples of such studies identified *TFPI2, RASSF2* and *SFRP2*, and *ITGA4* in fecal DNA (15). For gastric cancer, Reprimo was reported as a potential biomarker for early detection because only Reprimo was frequently methylated in both primary tissues and pair plasma samples (16).

The aims of the present study were as follows: (i) to confirm the correlation between DNA promoter hypermethylation and loss of gene expression in CRC cell lines; (ii) to confirm whether the demethylating agent 5-aza-2'-deoxycytidine (5-Aza-CdR) is able to restore gene expression; (iii) to identify which members of a panel of gene markers were most frequently hypermethylated in CRCs; (iv) to determine the correlation between methylation status and the clinicopathological features and survival; and (v) to determine whether the most frequently hypermethylated genes were methylated in bowel lavage and blood from venous drainage collected at the time of surgery.

Table I. *Summary data of genes tested.*

Gene abbreviation	Gene name	Gene location	Function	
3OST2	Heparen sulfate D-glucosaminyl 3-*O*-sulfotransferase-2	16p12.2	*O*-Sulfotransferase	(19)
CHFR	Checkpoint with forkhead and Ring finger domains	12q24.23	Mitotic stress checkpoint gene	(21)
RUNX3	Runt-related transcription Factor 3	1p.36.1	TGF-β signal pathway	(18)
p16	Cycline-dependent kinase inhibitor 2A	9p21	Cell cycle regulator	(22)

MSP primers

Gene	Forward primer	Reverse primer
3OST2	M:5'-CGGTTGTTCGGAGTTTTATC-3'	M:5'-GTAACGCTACCACGACCACG-3'
	U:5'-TGGAGTTTTATTGTTTAGGATT-3'	U:5'-AAAACTCACATAACACTACCACA-3'
CHFR	M:5'-ATATAATATGGCGTCGATC-3'	M:5'-TCAACTAATCCGCGAAACG-3'
	U:5'-ATATAATATGGTGTTGATT-3'	U:5'-TCAACTAATCCACAAAACA-3'
RUNX3	M:5'-TTACGAGGGGCGGTCGTACGCGGG-3'	M:5'-AAAACGACCGACGCGAACGCCTCC-3'
	U:5'-TTATGAGGGGTGGTTGTATGTGGG-3'	U:5'-ACACAACGCACTTAACCTATAA-3'
p16	M:5'-TTATTATGAGGGTGGGGCGGATCGC-3'	M:5'-GACCCCGAACCGCGACCGTAA-3'
	U:5'-TTATTATGAGGGTGGGGTGGATTGT-3'	U:5'-GATTTTGAATTGTGATTGTAA-3'

Materials and Methods

Cell lines. Eight CRC cell lines (LoVo, LS174T, DLD-1, SNU-C1, SW480, HCT116, COLO201 and RKO) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). They were grown in RPMI-1640 medium (Life Technologies, Rockville, MD, USA) supplemented with 5% fetal bovine serum and incubated in 5% CO_2 at 37°C.

RT-PCR for gene expression. Gene expression was analyzed by RT-PCR. Total RNA was extracted from cell lines with Trizol (Life Technologies), following the manufacturer's instructions. The RT reaction was performed on 2 μg of total RNA with Superscript II First-Strand Synthesis using the oligo (dT) primer system (Life Technologies). Primer sequences and conditions for RT-PCR product were as described previously (17-19).

The housekeeping gene *GAPDH* was used as an internal control to confirm the success of the RT reaction. Total RNA from human nonmalignant colonic epithelium (NME) was obtained from Clontech (Palo Alto, CA, USA). NME and normal peripheral blood mononuclear cells (PBMC) from healthy volunteers were used as controls for RT-PCR. PCR products were analyzed on 2% agarose gels.

5-Aza-CdR treatment. Cell lines with known gene promoter methylation were incubated in culture medium with the demethylating agent 5-Aza-CdR at a concentration of 4 μM for 6 days, with medium changes on days 1, 3, 5 and 9.

Clinical samples. Surgically resected specimens from 129 primary CRCs and corresponding NME were obtained from Department of Surgical oncology at the Gifu University hospital. Clinical staging data were available for all tumors: In addition, the most frequently hypermethylated genes were evaluated in bowel lavage and blood from the main drainage vein from 21 prospectively collected CRCs. Informed consent was obtained from the study individuals in accordance with protocols approved by the Institutional Review Board.

Bowel washing lavage and blood of the main drainage vein. DNA was extracted from bowel washing lavage as previously published (20).

When colorectal samples were resected, the intraluminal cavity which still contained CRC was washed with 100 ml saline, and then collected as lavage fluid. Next, the lavage fluid was centrifuged at 1000 rpm for 10 min to pellet the precipitate; finally, the supernatant was discarded and the pellet was used for extracting DNA.

The main drainage vein from the tumor-containing bowel was identified and as much as 2 ml (from 2.6 to 4.8 ml) of blood was collected.

DNA extraction and methylation-specific polymerase chain reaction (MSP). Genomic DNA was extracted from cancer tissue and corresponding NME from all tumor samples, lavage fluid and venous drainage blood using QIAmp DNA Mini Kit (Qiagen, Valencia, CA, USA). The extracted DNAs were bisulfite-treated using QIAmp Bisulfite Mini Kit (Qiagen, Valencia, CA, USA) to achieve maximal conversions and DNA recovery. Modified DNA was stored at –80°C until used. The methylation status of four genes (*RUNX3, 3OST2, CHFR* and *p16*) was determined by methylation-specific PCR (MSP) assays for cancer tissue and corresponding NME from each resection. References for methodology and gene information are summarized in Table I. PCR products were visualized on 2% agarose gels stained with ethidium bromide. DNA extracted from CRL 2577 cells (with known positive methylation status for all four tested genes) and then subjected to bisulfite treatment was used as a positive control for methylated alleles. Water blanks were included with each assay.

Data analysis. Frequencies of methylation of two groups were compared using the Students *t*-test. The relationship between hypermethylation and each clinicopathological features was analyzed using the χ^2 test and the Mann-Whitney *U* nonparametric test. Survival rates after operation were analyzed for each gene's methylation status using the Kaplan-Meier method, and the two survival curves were compared with the log-rank test. For these tests, probability values of $p<0.05$ were regarded as statistically significant.
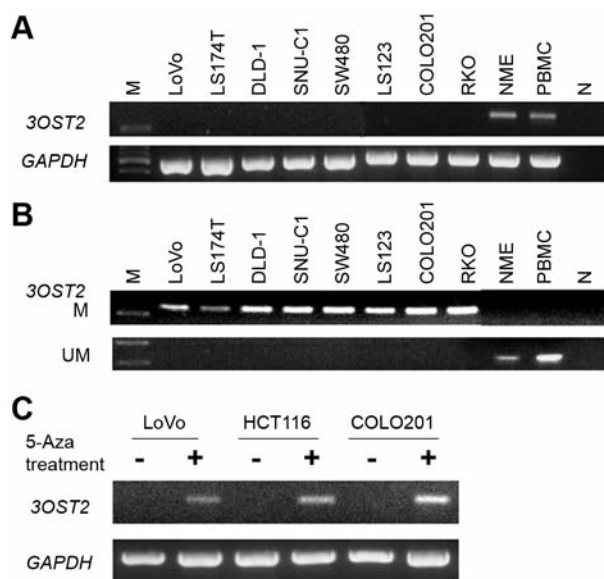
Figure 1. *Representative examples of RT-PCR (A) and MSP (B) of 3OST2 in CRC cell lines. Expression of the housekeeping gene GAPDH was run as a control for RNA integrity. NME, Nonmalignant colonic epithelium; PBMC, peripheral blood mononuclear cells; N, negative control (water blank). M, methylated form; UM, unmethylated form. In all instances, there was concordance between the presence of methylation and loss or down-regulation of expression. (C) Representative examples of the effect of 5-Aza-CdR treatment on restoring gene expression in 3OST2 methylated cell lines. +: With 5-Aza-CdR treatment; –: without 5-Aza-CdR treatment. Treatment with 5-Aza-CdR restored expression of 3OST2. Expression of the housekeeping gene GAPDH was run as a control for RNA integrity.*
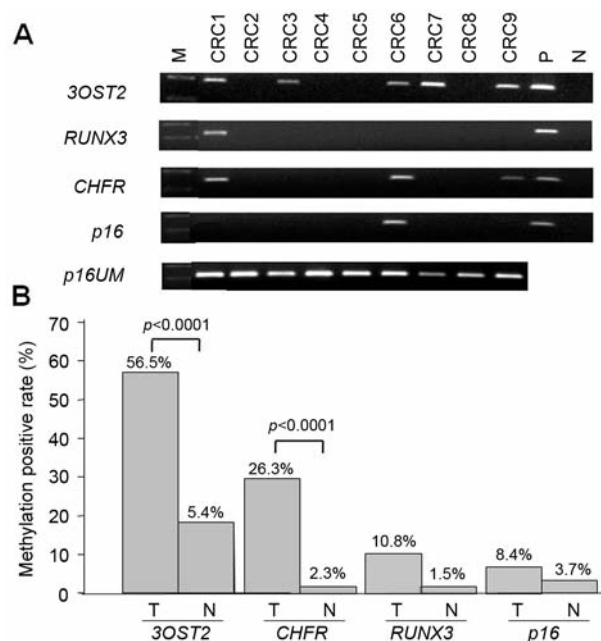

Figure 2. *A: Representative examples of MSP analyses of the methylated form of four genes in primary CRCs. The unmethylated form of p16 (p16UM) was run as an internal control for bisulfate treatment. B: Comparison of frequencies of aberrant methylation in CRC and NME samples. P-values are shown when there was a significant difference between two groups. T: Tumor tissue; N: normal tissue.*

Results

Aberrant promoter methylation and expression of RUNX3 (runt-related transcription factor 3), 3OST2 (heparan sulfate glucosamine 3-O-sulfotransferase 2), CHFR (checkpoint with fork head-associated and ring finger domains) and p16 in CRC cell lines. For four genes (*RUNX3, 3OST2, CHFR* and *p16*), the correlation between aberrant promoter methylation and loss of gene expression was examined using a panel of eight CRC cell lines. Aberrant methylation of *RUNX3, 3OST2, CHFR* and *p16* was found in five of eight, eight of eight, six of eight and six of eight lines respectively. Expression of the genes was examined by RT-PCR. The genes were expressed in NME and PBMC. However, loss or down-regulation of *RUNX3, 3OST2, CHFR* and *p16* gene expression was observed in 4/8, 8/8, 6/8 and 4/8 CRC cell lines, respectively. The concordance rates between loss of gene expression and aberrant methylation of these genes were 88% (*RUNX3*), 100% (*3OST2*), 100% (*CHFR*) and 75% (*p16*) (Figures 1A and B).

5-Aza-CdR treatment. Three cell lines for *RUNX3* (LoVo, DLD-1 and COLO201) and three cell lines for 3OST2 (LoVo, HCT116 and COLO201) that showed loss or down-regulation of expression and methylation by MSP were cultured with 5-Aza-CdR. Expression of these four genes was restored after treatment in all methylated cell lines tested (Figure 1C).

Frequency of methylation in primary CRCs and NME. Of 129 primary cases of CRC, 30 were Dukes A, 36 were Dukes B, 40 were Dukes C and 23 were Dukes D. They comprised 67 males (51.9%), with an average age of 63.5±13.1 years. Other clinical features of samples, such as tumor location and differentiation, are summarized in Table II. The methylation status of four genes in CRCs and NME was examined. The unmethylated form of *p16*, run as a control for DNA integrity, was detected in all of these samples. Representative examples of the DNA methylation analysis are shown in Figure 2A. Aberrant methylation of *3OST2, CHFR, RUNX3*, and *p16* (CRCs and NME) was found in 56.5%/5.4%, 26.3%/2.3%, 10.8%/1.5%, 8.4%/3.7%. *3OST2* was most frequently methylated in CRC samples. The methylation ratios were significantly higher in cancer tissues compared with healthy tissues in *3OST2* and *CHFR* genes.
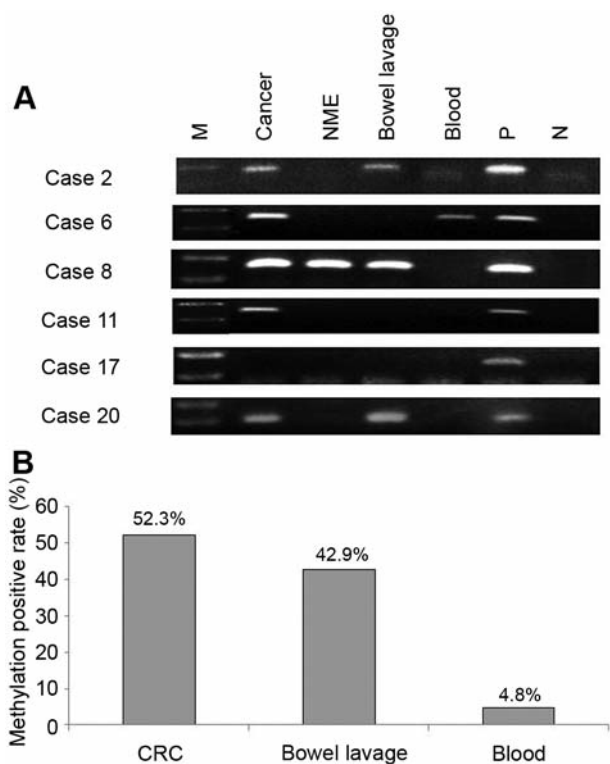
Figure 3. A. Representative examples of MSP analyses of methylated form of 3OST2 gene in primary CRCs , NME samples, bowel lavage and blood from tumor drainage vein. The unmethylated form of p16 (p16UM) was run as an internal control for bisulfate treatment. B. Comparison of frequencies of aberrant methylation of 3OST2 gene in primary CRCs, bowel lavage and blood from tumor drainage vein. Blood: blood from tumor drainage vein.

Table II. Clinicopathological features of all samples.

Age (years)	63.5	Dukes stage	
Gender		A	30
Female	62	B	36
Male	67	C	40
Location		D	23
Cecum	10		
Ascending colon	17		
Transverse colon	14		
Descending colon	3		
Sigmoid colon	32		
Rectum	53		
Differentiation			
Well	68		
Moderate	57		
Poorly	2		
Mucinous	2		

In contrast, there was no statistical difference between methylation-positive/-negative patient in *RUNX3* and *p16* genes (Figure 2B). Overall, 17/129 (13%) of the NME samples and 93/129 (72%) of the carcinomas were methylated in one or more of the four genes analyzed.

Correlation between methylation and other clinico-pathological features in CRCs. The correlation between methylation status and clinicopathological features including gender, age, TNM Stage, tumor location, depth of the tumor, lymph node metastasis and degree of tumor differentiation was examined. There was no correlation between any of these factors and methylation status of the genes. There was no statistical difference between methylation positive and negative groups for the survival curve after operation according to the methylation status of the four genes.

Frequency of 3OST2 methylation in bowel lavage and blood of main drainage vein. From the results of previously reported findings, 3OST2 became the candidate of the early detection for the CRCs. Next, using the DNA extracted from

bowel lavage and blood of main drainage vein, it was investigated whether early detection is possible by examining *3OST2* gene methylation status. Representative examples of the DNA methylation analysis of *3OST2* gene are shown in Figure 3A.

Regarding bowel lavage, *3OST2* gene was methylated in 42.9% (9/21) samples.

The *3OST2* gene was methylated in 73% (8/11) of cases *where* 3OST2 was methylated in primary cancers. There was excellent concordance between methylation status in tumors and lavage fluids (71.4%). The detection rate in blood of the main drainage vein was low (1/21 cases, 4.7%) (Figure 3B). Results are shown in Table III.

Correlation between 3OST2 methylation status of bowel lavage and clinicopathological features. The correlation between *3OST2* methylation status of bowel lavage and clinicopathological features including tumor size, depth of the tumor, TNM Stage and tumor location was examined. There was no correlation between any of these factors and *3OST2* methylation status of bowel lavage (Table IV).

Discussion

The *3OST2* gene encodes an *O*-sulfotransferase that is involved in the final modification step of glycosaminoglycan chains of heparin sulfate proteoglycans (HSPGs) (23). HSPGs are important in the field of migration, cell growth and adhesion due to their interactions with a wide range of growth factors, morphogens, cytokines and extracellular matrix (24). Silencing of the *3OST2* gene suggests that altered modification of HSPGs is involved in cancer development and progression in some cases (17). Methylation-associated silencing phenomenon of *3OST2* is

Table III. *3OST2 gene methylation status in bowel lavage and blood of main drainage vein.*

Case	Age (years)	Gender	Tumor location	TNM stage	Cancer tissue	NME	Bowel lavage	Blood
1	72	M	Sigmoid	3	U	U	U	U
2	77	M	Cecum	3	M	U	M	U
3	54	M	Ascending	1	M	U	M	U
4	80	F	Ascending	2	M	U	M	U
5	55	M	Rectum	1	U	U	U	U
6	81	F	Ascending	3	M	U	U	M
7	68	F	Ascending	1	U	U	U	U
8	39	M	Rectum	3	M	M	M	U
9	77	F	Cecum	3	M	U	U	U
10	80	M	Ascending	2	U	U	M	U
11	64	F	Ascending	1	M	U	U	U
12	45	M	Transverse	4	M	U	M	U
13	67	F	Sigmoid	2	M	U	M	U
14	43	M	Rectum	3	U	U	U	U
15	80	M	Sigmoid	2	M	U	U	U
16	78	F	Transverse	3	U	U	U	U
17	71	F	Ascending	3	U	U	U	U
18	75	F	Sigmoid	1	U	U	U	U
19	72	M	Transverse	3	U	U	U	U
20	79	M	Ascending	2	M	U	M	U
21	62	F	Sigmoid	1	U	U	M	

U: Unmethylated; M: methylated.

demonstrated in human breast, colon, lung and pancreatic cancers among others (19, 25).

In this study, *3OST2, RUNX3, CHFR* and *p16* were examined because it had previously been shown that *3OST2* is the most methylated and has the highest methylation rate of known genes (25). *RUNX3* was reported to be frequently methylated, especially in gastric cancer (18). In addition, frequent methylation of *RUNX3* has been reported in CRC cell lines (26). *CHFR* is a G_2/M checkpoint gene that has recently been described (27). It has received special attention because of its frequent inactivation in multiple different human cancer types. *p16* is a cyclin-dependent kinase inhibitor that acts like a negative regulator of cell growth and proliferation in the G_1 phase of the cell cycle (28). Interestingly, most studies have shown that the frequency of *p16* methylation is higher in colorectal tumors than in the corresponding normal mucosa (29, 30).

This study confirmed that the methylation status was correlated with gene silencing in CRC cell lines for *3OST2, RUNX3, CHFR* and *p16*. Moreover, the current results showed that expression of these genes was restored after 5-Aza-CdR treatment in CRC cell lines that did not express these genes, and which were hypermethylated. Therefore, it is confirmed that transcriptional repression of these genes was caused by hypermethylation.

Secondly, this study demonstrated that *3OST2* was the most frequently methylated of the four genes tested (*3OST2,*

Table IV. *Analysis of methylation status for 3OST2 gene in bowel lavage.*

	Methylation		
	(+)	(−)	*p*-Value
Tumor size			
<5 cm	5	8	
≥5 cm	4	4	0.60
Depth of the tumor			
T1,T2	2	2	
T3,T4	7	10	0.74
TNM Stage			
I, II	6	5	
III, IV	3	7	0.25
Tumor location			
caecum-transverse colon	3	6	
Descending colon-rectum	6	6	0.44

RUNX3, CHFR and *p16*). However, the findings of no significant correlation between hypermethylation of the *3OST2, RUNX3, CHFR* and *p16* and the clinicopathological features with the survival curve would support the observation that the aberrant methylation of genes occurs early in tumorigenesis in the gastrointestinal tract (9, 14). Consequently, this study further explored the potential of *3OST2* as a biomarker for the early detection of CRC using

bowel washing lavage and blood gathered from the main drainage vein. Because this second step is rather preliminary, bowel lavage and blood of the main drainage vein were used as surrogates for stool and peripheral blood.

A stool-based screening test for CRC has long been sought. Immunological fecal occult blood testing that uses antibodies specific to human hemoglobin is the most widely prescribed screening test for CRC because it is simple, noninvasive, and has been demonstrated to reduce mortality due to CRC. However, the analysis of fecal DNA represents an emerging new field for early detection of CRC (12, 14, 15, 31).

Thus far, the most reliable early detection of cancer is provided by a signature combining multiple gene mutations, each of which individually has a low sensitivity (32, 33). However, these methods may be more expensive and difficult to implement. Epigenetic events, which are seen more frequently than genetic events, may have the potential to achieve high sensitivity and specificity with a single gene (34, 35).

Detection of CRC-specific methylation in stool samples with a single epigenetic marker has also been explored recently. *TFPI2* methylation was detected in stool DNA from stage I to III CRC patients with a sensitivity of 76% to 89% and a specificity of 79% to 93% (31). Furthermore, *RASSF2* and *SFRP2* genes were successfully identified one or more methylated in fecal DNA from 75% of patients with colorectal cancer (n=152), but only in 10.6% of those without neoplastic or active disease (36). *ITGA4* from fecal DNA, another candidate for early detection, was demonstrated to be methylated in 75% of colon adenomas (n=36) and 92% of colon adenocarcinomas (n=75) (15). Regarding colonic lavage, Petko *et al.* demonstrated that analysis of colon lavage from patients who undergo colonoscopic exams revealed methylated *CDKN2A, MGMT,* and *MLH1* in fecal DNA from 31%, 48%, and 0% of individuals with adenoma and from 16%, 27%, and 10% of individuals with no detectable polyp, respectively (12).

This study demonstrated that, by examining the *3OST2* methylation status of the bowel lavage, 42.8% (9/21) of cases were methylated, with a sensitivity of 72.7% and a specificity of 90%, and it was rather less frequent than in cancer tissues. The DNA extracted from the bowel lavage-based assay had a lower methylation rate than cancer sample-based results (52.3%; 11/21). This may be due to the fact that the bowel lavage was collected without the use of a stabilization buffer and capture probes were not used to concentrate methylated *3OST2*, which may be technical problems that can be improved in future design. At least for bowel lavage there was some degree of concordance of methylation status between primary CRC and the bowel lavage. In addition, among nine cases with methylation-positive bowel lavage, early stage cases were in the majority

(two were stage I, four were stage II, two were stage III and one was stage IV), and in particular, the tumor depth of each single case was mucosal propria and submucosal layer.

Therefore, the Authors hypothesize that there might be a possibility of early detection for CRC by examining the bowel lavage, and then the stool in practical application. The *3OST2* gene was reported to be methylated in 67% of CRCs in a previous study (25). In addition, the current data regarding bowel lavage (42.8%: 9/21) and primary CRC (52.3%: 11/21) showed a relatively lower *3OST2* methylation rate than the former study. Consequently, there may be room for improvement of the methylation assay described in this study. One way of improving sensitivity and specificity may be to use real time PCR analysis (15).

As for methylation detection in circulating blood, Taback *et al.* showed that frequency of methylated DNA in CRC patients' mesenteric vein and peripheral vein was 0-19% and 0-6% (37). Recently, a PCR assay for analysis of *Septin* 9 (38) and a panel including *APC, MGMT, RASSF2A* and *Wif-1* genes (39) were reported to have a high sensitivity and specificity. In the former report, the sensitivity of the assay was 72% (90/125 CRCs detected), while maintaining 90% specificity (19/183 for controls). It was possible in this study to detect *3OST2* methylation in only 4.7% (1/21) in blood of the tumor drainage vein. One reason for this lack of diagnostic sensitivity may be insufficient DNA in the main drainage vein. Another reason for the suboptimal diagnostic sensitivity may be secondary to technical aspects related to the assay design. Whenever the application of methylation as a biomarker is considered, attention should be paid to the possibility that aberrant methylation of some tumor suppressor genes occurs after the onset of neoplastic evolution, and others become hypermethylated in normal epithelial cells from environmental factors, such as exposure to folate and aging. Thus, such factors might be the source of false-positives in the study of tumor-specific methylation in colorectal cancer (40, 41). This study showed that *3OST2* was methylated in 56.5% of primary CRC and 5.4% of NME. This study also showed that there was no correlation between methylation status and clinicopathologic features. Therefore, in this study, there was little possibility of such false-positives.

The present study is, to the Authors' knowledge, the first to examine the *3OST2* methylation status for the purpose of early detection of CRC in bowel wash and blood of drainage vein, and have potential clinical implications.

Because this study was limited by the small amount of bowel lavage and blood from the main drainage vein, additional work is needed to evaluate a larger number of such samples and to examine the methylation in stool from CRC patient to detect the methylation of the *3OST2* gene. In any case, *3OST2* enriches the list of currently studied epigenetic alterations in CRC and may be associated with a

noninvasive tumor test to monitor the general population. In conclusion, this study suggests that methylation of the *3OST2* gene may be a potential molecular marker for use in stool samples for the early detection of CRC.

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